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THE RELATIONSHIP OF INTESTINAL BACTERIA AND DIET COMPOSITION TO AMINO ACID REQUIREMENTS OF WHITE MICE

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Male, weanling, axenic mice were maintained under three conditions of intestinal flora on six diets. The results showed that mice with a conventional (normal) intestinal microflora required more lysine and methionine than their counterparts which were either germfree or inoculated with a limited flora. The effect was noticeable only when lysine and methionine levels were nearly at a minimum for growth of the mice.

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THE RELATIONSHIP OF INTESTINAL BACTERIA AND DIET COMPOSITION TO AMINO ACID REQUIREMENTS OF WHITE MICE

by

D. T. Munsey, Jr., W. Batchelder, A. Christiansen

Project Reference:
1T161102A71C

February 1975

U. S. Army Natick Laboratories
Natick, Massachusetts 01760

FORWORD

Many examples are on record of the effects, both detrimental and beneficial, of the intestinal bacterial flora on vitamin utilization by mammalian hosts. A rather limited amount of data is available on the effect of intestinal flora on amino acid utilization. This study is an attempt to add to the knowledge of flora effects on the amino acids l-lysine and l-methionine.

The work reported was conducted under Project No. 1T161102A71C, Food and Food Service Research.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council, and by the National Society for Medical Research.

ABSTRACT

Male, weanling, axenic mice were maintained under three conditions of intestinal flora on six diets. The results showed that mice with a conventional (normal) intestinal microflora required more lysine and methionine than their counterparts which were either germfree or inoculated with a limited flora. The effect was noticeable only when lysine and methionine levels were nearly at a minimum for growth of the mice.

TABLE OF CONTENTS

INTRODUCTION

For most of the twentieth century the intestinal microflora of man and lower animals has been considered influential in the overall nutrition and well being of the host. Since the time of Osborne and Mendel, in 1911, the importance of the intestinal microflora to host nutrition has been studied increasingly.

An important question in human nutrition is whether under disturbed conditions levels of one or more factors produced by the microflora can be reduced to the point of causing deficiencies. Microbes are probably indirectly related to requirements for all nutrients. It may even be possible in the future to modify the microflora to the host's advantage (1).

Although it has been established that the intestinal microflora plays an active role in vitamin synthesis and utilization in animals and, to a lesser extent, in man (2), very little has been published about the effect of the intestinal microbes on the utilization of amino acids.

Hötzel and Barnes (3), in their review in 1966, listed several studies on the subject of the intestinal microflora and amino acid utilization. Hall and Sydenstricker (4); Krehl et al. (5); Harper and Elvehjem (6); and Dubos and Schaedler (7, 8) show evidence for a definite influence by the intestinal microflora upon the utilization of amino acids. However, Harper and Katayama (9) felt their results indicated little, if any, effect of microflora.

Competition between the microflora and the host for amino acids was demonstrated by several investigators. For example, Sauberlich (10) showed that chlortetracycline and penicillin enhanced the growth of rats on low protein or amino acid deficient diets. The effect diminished with increasing protein levels suggesting that inhibition of the microflora enabled the host to compete better for the amino acids present. At the higher levels there was ample protein for both host and microbes. Donaldson (11) found that amino acid and calcium absorption by animals seemed increased by antibiotics. In addition, he found the small bowel flora could metabolize key products, for instance, bile acids, and cause steatorrhea. In 1960 Dubos and Schaedler (7) found their pathogen free (NCS) mice grew better than conventional mice on diets containing 15 percent wheat gluten as the sole protein source. When Escherichia coli was added to the intestinal flora of the NCS mice they responded like conventional mice. Rogers and Sarles (12) tested nineteen enterococcus

strains and found they required six of the nine amino acids essential for the rat. The microflora from a wild mouse (Mus muscularis) appeared to increase the requirement for lysine in the diet of laboratory mice from 0.4 to 0.6 percent of the dry weight of the diet (13).

Like the mouse, the human small intestine has been shown to contain a fairly constant, albeit small, bacterial flora in intimate association with the intestinal mucosa (11, 14-17). The organisms found are similar to those in the mouse including lactobacilli, bacteroides, anaerobic streptococci, coliforms, clostridia, and enterococci. It is probable that these organisms influence host nutrition through competition for nutrients, breakdown of digestive compounds like amino acids, bile acids, and by influencing histological development and function of the gut wall (18). This paper describes further studies of the effects of the intestinal bacterial flora on the utilization of, and requirement for, the amino acids l-lysine and l-methionine.

MATERIALS AND METHODS

Twenty-one-day-old, male, CD-1 strain, axenic mice from the Charles River Mouse Farms, Inc., Wilmington, Mass. were employed. In each test group there were twelve mice making a total for each experiment of 216 mice. All mice were housed in clear plastic cages on stainless steel grids. The cages were kept inside flexible plastic isolators of the Trexler type (19). The isolators were sterilized with 4.0% peracetic acid in water with a small amount of sodium alkyl lauryl sulfate added as a wetting agent. Routine sterilization of air locks and material entering the isolators was done with 2.0% peracetic acid.

The diets were modeled after the agar gel type used by Rogers and Harper (20). They were developed by Chen et al. (21) to counteract unfavorable osmotic effects seen with dry diets containing crystalline amino acids. Diet composition was modified somewhat (table 1) to suit our needs.

The complete diets were sealed in cans at atmospheric pressure, frozen to -60°C, and then sterilized by exposure to 4.0 Mrad in a ^{60}cobalt source. After sterilization, the diets were kept at room temperature. Water and food were available ad libitum.

The bacterial cultures consisted of the cecal contents of two conventional COBS CD-1 strain mice from Charles River Mouse Farms, Inc., and six strains known collectively as Schaedler's flora[1] (22). The cecal organisms were the conventional flora.

[1]Kindly supplied by Dr. R. W. Schaedler, then at Rockefeller Inst., N.Y.

The organisms inoculated into the mice were as follows:
Schaedler's flora:

1. a slow lactose fermenting coliform

2. an enterococcus

3. a bacteroides

4. a rhizoid* lactobacillus

5. a compact* lactobacillus

6. a group N streptococcus

Conventional flora

1. A lactose fermenting coliform

2. a non lactose fermenting coliform

3. a staphylococcus

4. an enterococcus

5. a lactobacillus

6. a clostridium

7. a bacteroides

The main differences between the two flora are the coliforms and the presence of a staphylococcus and a clostridium in the conventional flora.

The mice were inoculated by placing cotton swabs soaked in appropriate broth cultures against their mouths. They invariably bit the swab and inoculated themselves. Fecal samples were collected during the experiment to check the presence of the test organisms in the animals. All these organisms were recovered from the feces of the test mice by homogenizing the feces in Gall's broth (23) and plating appropriate serial 10-fold dilutions on selective media.

* Refers to colonia morphology

Ear notching identified the mice and each was weighed on day 1 for a baseline. They were weighed once a week for three weeks and sacrificed. Mouse weight gains were considered to be directly related to amino acid utilization.

RESULTS

The average weight gains for the lysine and methionine groups of mice are shown in tables 2-4. The average represents 8-12 mice with the exception noted by the number in parentheses.

The 0.2% lysine and 0.25% methionine produced the third best growth, and the 0.0% lysine and the 0.0% lysine and methionine produced little or no growth in any group. In the methionine experiment, the soy protein (0.47% methionine), 0.5% and 0.75% methionine were equal in growth production. No differences in growth were found between germfree mice and mice inoculated with Schaedler's flora. Both groups grew better than the mice inoculated with the conventional flora.

Regardless of flora, the casein diet produced the best growth of all diets. The analysis of co-variance found no difference among means for the gluten, 0.4, and 0.8 percent lysine diets which produced the next best growth.

DISCUSSION AND CONCLUSIONS

The results indicate that mice inoculated with conventional flora require more lysine and methionine than germfree mice or mice inoculated with Schaedler's flora. This was observed only when the concentrations were 0.2 percent and 0.25 percent or lower, for lysine and methionine, respectively. Since the only known difference among these mice was their intestinal microflora, the different requirements found are presumed to be caused by the flora. Further support for the role of the bacterial flora comes from Donaldson (11) who found that amino acid absorption was increased by antibiotic treatment. Unfortunately, it was not clear whether absence of bacteria or presence of antibiotics caused the increase. The reduced gain of mice inoculated with a conventional flora when fed gluten as their protein source agrees with the work of Dubos and Schaedler (7). Their NCS mice, which had a limited bacterial flora, were able to grow normally on gluten as were our limited microflora mice.

Analysis of the data showed that weight gains during the test period were dependent upon the starting weights of the mice. In other words, the heavier a mouse was at the start of a test, the less he would gain irrespective of diet. (See table 2.) The analysis of

covariance technique was employed to nullify the effect of any differences in starting weight. That is why in several instances weight gains which appear identical are statistically different at the 5 percent level of probability.

The human small intestine contains a fairly constant population (10^3 - 10^4 org/g) of microorganisms including populations of streptococci (viridans), lactobacilli, fungi, staphylococci, and occasionally, fusiforms viellonella, and clostridia (11, 4, 15). These organisms have been shown to be in intimate association with the intestinal mucosa (11). Kalser et al. (16) found E. aerogenes, E. coli and alpha streptococci in 50 percent of human jejunal samples taken by aspiration. They also found anaerobic lactobacilli.

The coliforms and clostridia found probably account for most of the influence exerted on host nutrition because their greater range of biochemical activity demands more compounds in competition with the host (24). Rogers and Sarles (12) found that 19 strains of enterococci require 6 of the 9 amino acids essential for the rat. This suggests a link between human nutrition and microflora of the small intestine which could well be influential in persons eating marginal diets.

In addition, there are some physiological changes which may influence host nutrition which are related to intestinal microflora. Intestinal organisms may cause mild toxemias which decrease efficiency of food utilization. There are some major differences between germ free mice and mice with a conventional flora which may influence their utilization of nutrients and consequently their growth rate. Among these are oxidation-reduction potential of germfree rat ceca which is 200-300 millivolts more positive than that of mice with a conventional microflora (25). Other work (26) showed that DL-tryptophan -3-C^{14}, L-tryptophan -H^3, and C^{14} labelled algal protein acid hydrolysate are absorbed twice as fast in germfree mice as in mice with a conventional flora. It also appears that the intestinal microflora may interfere with absorption of nutrients by thickening the gut wall (18).

It seems reasonable to conclude that the human intestinal microflora could influence host nutrition. A number of organisms in the human small intestine have been shown to influence absorption of amino acids and other nutrients by either competition or physical changes.

If the microflora influences the hosts nutrition in humans to the degree it does in mice, nutritional problems would only be noticeable when the host is eating a diet of nutritionally marginal quality.

TABLE 1

Basal Diet (To Which Various Amino Acid Sources Were Added)

Component	% of Dry Wt
Lard (Armour)	7.0
Corn oil (Mazola)	3.0
R - H salt mix (General Biochemical Corporation	4.0
Choline chloride (50% aqueous solution)	0.4
Glucose (added in about equal amounts to make from 59.6 - 63.7 percent of dry ingredients, depending on amount of protein added)	
Sucrose	
Dextrin	
Vitamin mix[a]	1.0
Agar [b]	4.0

Amino Acid Sources Added to Basal Diet as Appropriate
(given in percent of dry wt)

A.A. Source	Casein	Gluten	0.0%	0.2%	0.4%	0.8%
Lysine Experiment						
Vitamin-free casein	22.0	X	X	X	X	X
Wheat gluten	X	22.0	X	X	X	X
Amino acid mix[c]	X	X	17.06	17.06	17.06	17.06
l-lysine	X	X	0.0	0.2	0.4	0.8
Methionine Experiment						
Vitamin-free casein	21.0	X	X	X	X	X
Soy protein	X	21.0	X	X	X	X
Amino acid mix	X	X	16.86	16.86	16.86	16.86
l-methionine	0.3	X	0.0	0.25	0.50	0.75

[a] As given in Dymsza et al. (27).
[b] Agar added to boiling water. Water equal in weight to total of dry ingredients. The agar and water were added while the suspension was still molten.
[c] AA mix same as best mix in Rogers and Harper (20) (lysine or methionine and cystine omitted where appropriate) except glutamate, 4.50%; glycine, 1.56%; proline, 1.00%; asparagine, 1.00%.

TABLE 2

Average Weights of Mice (g) for Each Diet and Flora Condition

Lysine Content Varied

Diet[a]	Germfree			Schaedler			Conventional		
	Initial	Final	Gain	Initial	Final	Gain	Initial	Final	Gain
Cas	27.3	34.8	7.5	25.7	34.3	8.6	25.7	35.5	9.8
Glu	26.6	34.0	7.4	18.8	27.1	8.3	22.0	27.9	5.9
0.0	23.2	23.9	0.7	17.2	16.8	-0.4	16.4	14.8	-1.6
0.2	25.4	31.5	6.1	23.7	29.1	5.4	19.0	21.7	2.7
0.4	11.5	20.8	9.3	20.4	30.4	10.0	24.9	31.9	7.0
0.8	24.8	32.2	7.4	24.9	31.9	7.0	27.6	30.5	2.9

Methionine Content Varied

Diet[a]	Germfree			Schaedler			Conventional		
	Initial	Final	Gain	Initial	Final	Gain	Initial	Final	Gain
Cas	21.7	34,5	12.8	14.8	30.6	15.8	16.2	26.9	10.7
Soy	20.3	32.9	12.6	14.7	29.0	14.3	15.9	26.2	10.3
0.0	14.7	12.5	-2.2	10.9	8.4	-2.5	13.2	11.0(2)[b]	-2.2
0.25	18.8	29.8	11.0	15.0	24.9	9.9	16.2	21.3	5.1
0.50	18.3	32.4	14.1	19.3	31.8	12.5	19.7	29.7	10.0
0.75	21.2	33.4	12.2	18.0	31.0	13.0	19.8	30.6	10.8

[a] See table 1.
[b] Only two mice survived to the finish in this group.

TABLE 3

The Effect of Intestinal Microflora with Different

Diet Levels of l-Lysine on Mouse Growth

Diet	Mouse Wts in Grams Gained[a] Intestinal Flora			Mean Wt Gain by Diet
	Germfree	Schaedler	Conventional	
Casein (1.5% lysine)	7.5	8.6	9.8	8.6 (a)
Wheat gluten (0.39% lysine)	7.4	8.3	5.9	7.2 (a)
0.0% l-lysine	0.7	-0.4	-1.6	<0 (c)
0.2% l-lysine	6.1	5.4	2.7	4.8 (d)
0.4% l-lysine	9.3	10.0	7.0	8.8 (b)
0.8% l-lysine	7.4	7.0	2.9[b]	5.8 (b)
Mean wt gains by flora	7.5(x)	7.9 (x)	5.7(y)	

Numbers bearing same superscript letter in a given row or column not significantly different at P = 0.05.

[a] Mice weighed at 39, 49, and 59 days of age.

[b] Figure is low due to heavy starting weight of mice. See Table 2.

TABLE 4

The Effect of Intestinal Microflora with Different Diet Levels of 1-Methionine on Mouse Growth

Diet	Mouse Wts in Grams Gained[a] Intestinal Flora Germfree	Schaedler	Conventional	Mean Wt Gain by Diet
Casein (0.65% methionine and cystine)	12.8	15.8	10.7	$13.1^{(a)}$
Soy protein (0.47% methionine and cystine)	12.6	14.3	10.3	$12.4^{(b)}$
0.0% 1-methionine	-2.2	-2.5	-2.2	$< 0^{(c)}$
0.25% 1-methionine	11.0	9.9	5.1	$8.7^{(d)}$
0.50% 1-methionine	14.1	12.5	10.0	$12.2^{(b,e)}$
0.75% 1-methionine	12.2	13.0	10.8	$12.0^{(b,e)}$
Mean wt gains by flora	$12.5^{(x)}$	$13.1^{(x)}$	$9.4^{(y)}$	

Numbers bearing same superscript letter in given column or row not significantly different at P = 0.05.

[a] Mice weighed at 31, 37, 43, and 49 days of age.

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